Evaluation of Antibacterial and Wound Healing Activity of *Hibiscus micranthus* Linn.

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As members of examining board of the final MSc. open defense, we certify that we have read and evaluated the thesis prepared by Berhan Begashaw entitled "Evaluation of Antibacterial and Wound Healing Activity of *Hibiscus micranthus* Linn*."* and recommended that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Pharmacology.

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I hereby certify that this thesis entitled "Evaluation of Antibacterial and Wound Healing Activity of *Hibiscus micranthus* Linn." of Mr. Berhan Begashaw, conducted under my supervision, is based on the original research work. It is certified that the material from other sources referred to in this MSc. thesis has been duly acknowledged in the list of references/ bibliography and that there is no infringement /violation of the provisions of patent and copyright while incorporating material derived from other sources.

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Declaration of Investigator

This is to certify that the thesis entitled "Evaluation of Antibacterial and Wound Healing Activity of *Hibiscus micranthus Linn.*" has been prepared and submitted under the supervision of Dr. Bharat Mishra and Asegedech Tsegaw and it describes the original research work done by me, in the Department of Pharmacology, School of Pharmacy, College of Medicine and Health Sciences, University of Gondar, Ethiopia for the degree of approval of Master of Science in Pharmacology.

To the best of my knowledge and belief, this work has not been carried out elsewhere and all the sources of materials used for this thesis have been duly acknowledged.

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ABBREVIATIONS/ACRONYMS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
Conc. H_2SO_4	Concentrated sulfuric acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
E. faecalis	*Enterococcus faecalis*
E. coli	*Escherichia coli*
FDA	Food and Drug Administration
H.micranthus	*Hibiscus micranthus*
IAEC	Institutional Animal Ethical Committee
K. pneumoniae	*Klebsiella pneumoniae*
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentrations
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCCLS	National Culture Collection Laboratory Standard
NFZ	Nitrofurazone
OECD	Organization for Economic Cooperation and Developmen
P. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*
S.E.M	Standard error of mean
S. pneumoniae	*Streptococcus pneumoniae*
SPSS	Statistical Package for Social Sciences
S. pyogenes	*Streptococcus pyogenes*
WHO	World Health Organization

ABSTRACT

Background: Infectious diseases are the most common causes of morbidity and mortality in developing countries. Now a day, medicinal plants play a major role in treatment of infectious diseases and for wound healing due to their secondary metabolites with minimum side effects. They are easily available and more affordable as compared to synthetic compounds.

Objectives: To investigate the antibacterial and wound healing activities of 80% methanol extract of *Hibiscus micranthus* leaves, the traditionally claimed medicinal plant**.**

Experimental design: Fresh matured leaves of *H.micranrhus* was collected, washed, dried under shade, crushed and extracted. *In vitro* antibacterial screening against *S. aureus, S.pneumoniae, S. pyogenes, E. coli, P. aeruginosa, K. pneumoniae* and *P. mirabilis* bacterial strains using disc-well diffusion assay were conducted. Animals of either sex were randomly divided into four groups each consisting of six rats. Group I (ointment base), Group II served as a positive control (NFZ 0.2% ointment), Groups III and IV (5 and 10% extracts) for *in vivo* wound healing activity by excision wound model, respectively. The acute oral toxicity test and skin sensitivity test were also performed before conducting the actual study. The extract was analyzed for secondary metabolites using standard methods.

Results: The leaves extract exhibited varying degrees of sensitivity with zones of inhibition ranging from 14.00±0.333 (*S.pyogenes*) to 22.67±1.202 (*S.aureus*). It was found that *S. aureus* and *S. pneumonia* (*p<0.05)* were the most sensitive to the extracts of the leaves at concentrations of 800µg/ml and 400µg/ml, respectively followed by *P. aeuruginosa* [(18.33±.333mm) *(p<0.05)*] at a concentration of 400µg/ml. However, *E. coli* and *P. mirabilis* were found to be resistant to the extract at any of the applied doses. The MIC was found to be 0.625, 2.5, 1.25 and 5mg/ml for the extract against *S. pneumoniae, S. aureus, P. aeuruginosa* and *K. pneumonia,* respectively. *S. aureus, P.aeuruginosa* and *K.pneumoniae* had MBC value of 5mg/ml, while *S. pneumoniae* had MBC value 1.25mg/ml. In the wound healing study, the 5 and 10% w/w extract exhibited significant wound contraction rate of 99.30% and 99.13% as compared to NFZ ointment and simple ointment base treated groups from 6^{th} to 16^{th} day, respectively ($p<0.05$). Complete healing was produced at the 16^{th} day when extract ointments were used. Alkaloids, flavonoids, saponins, tannins, steroids, phenols, diterpines and anthraquinones were found to be present in the extract.

Conclusion: The present study suggests that the methanol extract of the leaves exhibited a potential antibacterial activity against the tested microorganisms which may be attributed to the presence of phytoconstituents like alkaloids, flavonoids, saponins, tannins, steroids, phenols and diterpines and wound healing activity which may be attributed to the presence of flavonoids, saponins, tannins, phenols, diterpines and anthraquinones to the extract. The extract inhibited both gram positive and gram negative organisms indicating that it has broad spectrum of antibacterial activity.

Key words: *Hibiscus micranthus* Linn., antibacterial activity, wound healing activity, excision wound

1. INTRODUCTION

1.1. Infectious diseases

Infectious diseases are the most common causes of morbidity and mortality in developing countries [1]. Deaths from infectious diseases occur disproportionately in the developing world, where they are the biggest killer of children and young adults [2]. Each year, these diseases kill almost 9 million people many of them are children under five. Stepping up according to the latest published data in 2012, infectious diseases were responsible for the death of more than 8.7 million people worldwide in 2008 [3]. Most of the common diseases in Africa are infectious due to infection by living organisms (bacteria, viruses, parasites or fungi) because of poor socio-economic status of the individuals makes them susceptible to a variety of diseases, low educational status and lack of access to modern health care service [4]**.** To control infectious diseases, antibiotics are used which represent approximately 30% of acute care hospitals during expenditure and they are prescribed for 20-50% of inpatients. Use of antibiotics has contributed to the dramatic fall in morbidity and mortality from communicable and infectious diseases over the last 50 years globally. However, the control of infectious diseases is seriously threatened by the steady increase in the number of microorganisms that are resistant to antimicrobial agents. Emergence of antimicrobial resistance is the result of the use, over use and misuse of antibiotics [1]. John *et al*., (2011) described that the widespread use of broad spectrum antibiotics has lead to the emergence of several resistant strains of microbes. These contribute significantly towards rise in the escalating health care costs and patient morbidity and mortality [5].

The incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) among hospitalized subjects is approaching 60% with increasing prevalence of vancomycin- intermediate *S. aureus* and community-associated MRSA. According to Infectious Disease Society of America, the incidence of vancomycin-resistant *Enterococcus* infections is reached around 30% and the incidence of fluoroquinolone-resistant *Pseudomonas aeruginosa* infections exceeds 30% [6]. There have been only seven systemic antibiotics approved by the Food and Drug Administration (FDA) between 1998 and 2002, five between 2003 and 2007 and two between 2008 and 2010 [6]. Although it is expected that microorganisms will eventually develop resistance to available antimicrobials, the rate at which they are developing resistance far outweighs our current ability to develop new antimicrobials [7].

There are a number of ways by which microorganisms are resistant to antimicrobial agents including enzymatic inhibition of drugs, alteration of proteins targeted by antibiotics, changes in metabolic pathways, antibiotic efflux and alterations in porin channels and changes in membrane permeability [8, 9]. So, these are an urgent need of the use of new drugs obtained from few plants to control a condition called "antibiotic crisis".

1.2. Wound and its pathology

Wound is defined as disruption of cellular, anatomical and functional continuity of a living tissue. It may be produced by physical, chemical, thermal, microbial or immunological insult to the tissue. When skin is torn, cut or punctured it is termed as an open wound and when blunt force trauma causes a contusion it is called closed wound, whereas the burn wounds are caused by fire, heat, radiation, chemicals, electricity or sunlight. In other words, wound is a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of underlying normal tissue and may also result from a contusion, hematoma, laceration or an abrasion [10]. Wound infections are most common in developing countries because of poor hygienic conditions [11, 12]. Pattayanac and Sunita (2011) and EWMA (2005) described that *S. aureus, S. pyogenes, E. coli, P. aeroginosa, S. pneumoniae* and *K. pneumoniae* are some important organisms causing wound infection [11, 12].

1.2.1. Classification of wounds

Wounds can be classified according to various criteria. Time is an important factor in injury management and wound repair. Thus, wounds can be clinically categorized as acute and chronic according to their time frame of healing [13].

1.2.1.1. Acute wounds

Wounds that repair themselves and that proceed normally by following a timely and orderly healing pathway with the end result of both functional and anatomical restoration are classified as acute wounds and can be acquired as a result of traumatic loss of tissue or a surgical procedure. According to the review done by Valnar *et al*., (2009) and Robson *et al*., (2001), the time course of healing usually ranges from 5 to 10 days or within 30 days [13, 14].

1.2.1.2. Chronic wounds

Valnar *et al*., (2009) and Robson *et al*., (2001) also described that chronic wounds are those that fail to progress through the normal stages of healing and they cannot be repaired in an orderly

and timely manner [13, 14]. The healing process is incomplete and disturbed by various factors which prolong one or more stages in the phases of haemostasis, inflammation, proliferation or remodeling. These factors include infection, tissue hypoxia, necrosis, exudates and excess levels of inflammatory cytokines. Chronic wounds may result from various causes including naturopathic, pressure, arterial and venous insufficiency, burns and vasculitis [13].

1.2.2. Complicated wounds

According to Robson *et al*., (2001), a complicated wound is a special entity and is defined as a combination of an infection and a tissue defect [14]. Other criteria taken into account during wound classification include etiology, degree of contamination, morphological characteristics and communication with hollow or solid organs [13, 14]. Etiology classifies wounds according to the trigger factor into contusions, abrasions, avulsions, lacerations, cuts, stab wounds, crush wounds, shot wounds and burns. According to the degree of contamination, wounds are classified in to aseptic wounds (bone and joint operations), contaminated wounds (abdominal and lung operations) and septic wounds (abscesses and bowel operations) [13]. Shrimanker *et al.*, (2013) and Andrew *et al*., (2009) generalized that all wounds have the potential to become chronic if the treatment regime is incorrect or inappropriate [10, 15].

1.2.3. Wound healing process

Wound healing is an orderly process of repair that follows injury to the skin and other soft tissues or it is a complex and protracted process of tissue repair and remodeling in response to injury [16, 17]. Wound healing involves a complex interaction between epidermal and dermal cells, the extra cellular matrix, controlled angiogenesis and plasma-derived proteins all coordinated by an array of cytokines and growth factors. Shrimanker *et al*., (2013) and Midwood *et al*., (2004) described that wound healing occurs at the stages of inflammation, proliferation and remodeling [10, 18].

In the inflammatory phase, bacteria and debris are removed through a process of phagocytosis and factors that cause the migration and division of cells involved in the proliferative phase are released.The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction. In angiogenesis, new blood vessels grow from endothelial cells. In fibroplasia and granulation tissue formation,

fibroblasts grow and form a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin [10, 18].

During epithelialization, epithelial cells cover the wound bed. During wound contraction, the wound reduces in size by the action of myofibroblasts, which establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. In the maturation and remodeling phase, collagen is remodeled and realigned along tension lines. This process is often disrupted leading to chronic and/or delayed wound healing [19]. Healing skin wounds proceeds in accordance with the mentioned below stages (FIGURE 1) [20].

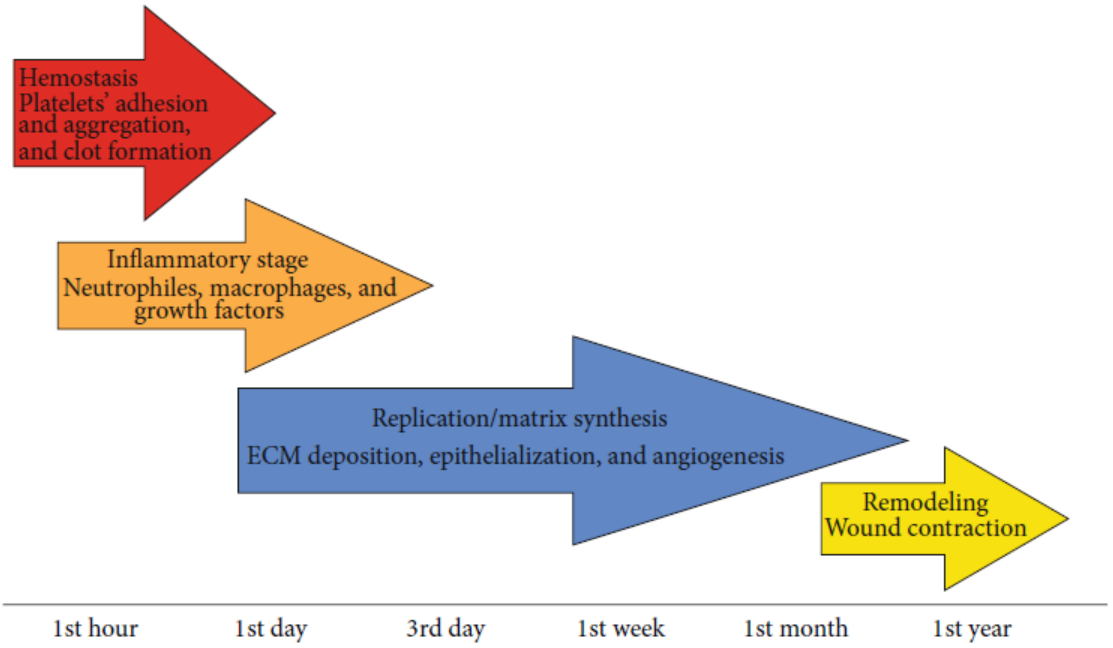

FIGURE 1: Healing stages.

Source: Pawel *et al*., 2014

1.2.3.1. Healing by primary and secondary intention

Successful wound healing depends on the timely and optimal functioning of many diverse processes, cell types, molecular mediators and structural elements. Healing by primary intention occurs when the wound edges are brought together by sutures, clips, staples or glue. There is often minimal tissue loss and the healing process is relatively short [10, 15]. In secondary intention healing, the wound edges cannot be easily brought together, usually due to a loss of tissue or infection. Thus, there is an open wound, occasionally a cavity, which heals from the base of the wound and in the latter stages by contraction of the wound edges [9, 15].

1.3. Management of infectious disease

1.3.1. Management with modern drugs

According to Mandell *et al*., (2010), substances with anti-infective potential have been applied medically for thousands of years [21]. Antimicrobial agents may interfere with cell wall synthesis (beta lactams: penicillins and cephalosporins and vancomycine), inhibit protein synthesis (tetracyclines and aminoglycosides by binding to the 30S ribosomal subunit) and **(**macrolides and chloramphenicol by binding to the 50S ribosomal subunit), interfere with nucleic acid synthesis (fluoroquinolones and rifampin) or inhibit a metabolic pathway for folic acid synthesis (sulfonamides and trimethoprim). The modes of action of antimicrobial agents against gram-positive and gram-negative bacteria are very similar [9, 21].

Ampicillin is amino penicillin that acts on bacteria by interfering with the transpeptidation reaction of bacterial cell wall synthesis. It is active against non–β-lactamase producing gram-positive bacteria, anaerobes, gram-negative *cocci* and *Enterobacteriaceae*. However, it is not active against *Klebsiella, P. aeruginosa* and indole-positive *Proteus* species [21]. Ciprofloxacin is a second generation fluouroquinolone that blocks bacterial deoxyribonucleic acid (DNA) synthesis by inhibiting bacterial topoisomerase II (DNA gyrase) and topoisomerase IV. It possesses excellent gram-negative activity and moderate to good activity against gram-positive bacteria [21].

1.3.2. Management with plant medicines

Now a day, medicinal plants also play a major role in treatment and managing infectious disease due to their secondary metabolites. Several studies showed that many medicinal plants have antibacterial [22-27] and wound healing activity [19, 28].

Rahimifard *et al.,* (2014) showed that the essentials from *Heliotropium bacciferum* extracts exhibited a significant activity against *S. aureus, Bacillus cereus, P. aeruginosa, E.coli* and *Salmonella enteritidi* strains [22]. Yadave *et al.,* (2011) found that different extracts of roots of *Rumex nepalensis* showed antibacterial activity against *S.aurues, S.mutans, E.coli* and *P. aeruginosa* [23]. According to Xavier and Rajeshwari (2012), bark extract of *Acacia concinna* possessed potential broad spectrum antibacterial activity [24]. Ibrahim *et al*., (2012) also found that the methanol extract of *Solanum nodiflorum* effectively inhibited the growth of *S. aureus, Bacillus cereus, Salmonella paratyphi* A, *P. aeruginosa* and *Proteus mirabilis* [25].

According to the Kutema *et al., (2013),* the methanolic extract and aqueous fraction of leaf and stem bark of the *Sclerocarya birrea* confirmed a broad spectrum of activity against *E. coli, P. aeruginosa* and *S. aureus* [26]. Sohel A.*et al*., (2010) also demonstrated that the flower extracts of *Hibiscus rosa-sinensis* had stronger antibacterial effects [27].

The research done by Ezike *et al*., (2009) showed that the 5 % and 10% of extract of *Phyllanthus niruri* significantly reduced the wound diameter producing 90.9 and 93.7% wound contraction respectively on day 18 post wounding, epithelialization time of excised wounds, increased the rate of wound closure and significantly increased the weight of granuloma tissue [28]. Shivanada *et al*., (2007) has demonstrated that an ethanol extract of *Hibiscus rosa sinensis* has properties that render it capable of promoting accelerated wound healing activity [19].

1.4. Management of wounds

Once a diagnosis of wound infection has been confirmed and antibiotic sensitivities identified, appropriate management regimens should be considered with a high priority given to reducing the risk of cross infection. Antimicrobial therapy is used only after a comprehensive assessment process including consideration of patient characteristics, the results of microbiological investigations and the identification of both the nature and location of the wound can the most appropriate antibiotic is identified. The main treatment objective will be to reduce rather than eradicate the bacterial burden within the wound margins. In addition to antimicrobial therapy, described by Mark (2004), there are also two main generic groups of wound management products that have the potential to reduce the bacterial burden in the wound those are compounds containing silver or iodine [29].

European Wound Management (EWMA, 2006) noted that, the use of newer formulation topical antimicrobials, particularly silver and iodine products are increasingly recommended as one component of the management of wounds with a problematic or increasing bacterial burden [30]. Iodine has antiseptic properties and active against a number of pathogens. In wound management, iodine is used in two forms: Cadexomer iodine – containing 0.9% elemental iodine that is released on exposure to wound exudates and may also be able to accelerate wound healing**.** PVP-1 (Povidone iodine) - an iodophor composed of elemental iodine and a synthetic polymer. Clinically, iodine is indicated for wound cleansing, wound bed preparation and the prevention and management of wound infection [29].

Silver interferes with the bacterial electron transport system and inhibits the multiplication of the bacteria. The chemical bonding of silver with a sulphonamide antimicrobial - sulphadiazine - has resulted in the development of a safe broad-spectrum agent for topical use (eg. flamazine). In this formulation, silver is released slowly from the transport medium in concentrations that are selectively toxic to micro-organisms such as bacteria and fungi. This type of silver product has been used successfully in the management of acute and chronic wounds [29]. There is also evidence that silver may have anti-inflammatory properties [30].

It is noted by Gerrard and Jesus (1983), nitrofurazone has been used to treat surface wounds involving all parts of the body and all kinds of injuries. It has enjoyed in the treatment of wounds because of its bactericidal properties and its effectiveness against a wide range of gram positive and gram-negative organisms. It is important to note that nitrofurazone is used topically and therefore does not interfere with concomitant systemic therapy [31].

1.5. Plant Medicines

The world is rich with natural products including medicinal plants. Medicinal plants are now getting more attention than ever because they have potential of myriad benefits as a source of drugs to all mankind. The medicinal value of these plants lies on bioactive phytochemical constituents that produce definite physiological action on the human body. Some of the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoid, saponins, phenolic compounds and many more. These natural compounds are the foundations of modern drugs as we know today [32].

According to WHO, about 80% of the population of the world depends on traditional medicine, mostly herbal remedies, for their primary health care needs [33]. Nasir *et al*., (2011) reported that the African continent has a long history with the use of plants and in some African countries up to 90% of the population relies on medicinal plants as sources of drugs [33]. There are approximately 60,000 plant species in Africa, which represents roughly a quarter of the world plant population. Despite the vast wealth and endemicity of the Africa plant biodiversity and associated cultures, Africa has only contributed 83 of the world‘s 1100 leading commercial medicinal plants [34]. Out of a population of 700 million of sub Saharan Africa, mentioned by Dharani (2010), only less than 30% have access to modern health care and pharmaceuticals [35].

According to Demisew and Dagne (1998) report, about 80-95% of traditional medicine preparations in Ethiopia are mentioned to be of plant origin [36].

In the 21st century, traditional medicine is gaining importance in mainstream healthcare since most people seek relatively safe remedies and approaches to healthcare. The need for herbal medicines, herbal health products, herbal pharmaceuticals, food supplements and herbal cosmetics is increasing globally due to the growing recognition of these products as they are thought to be non-toxic, have fewer side effects, good compatibility with physiological flora and are easily available at affordable prices [37]. Studies assessed by Khan *et al*., (2014) also claimed that some plants which are already used as traditional medicine possess antimicrobial properties and preparation from such plants are considered to be effective against diseases of microbial etiology like small pox, tuberculosis, typhoid, diphtheria and etc [38].

1.5.1. *Hibiscus micranthus* Linn.

Hibiscus a genus in the family of malvaceae encompasses more than 300 species [39]. *H. micranthus* (malvaceae) is a shrub up to 3 meter, stem erect, branched, usually with stiff, slender and stellately hairy plant as shown in Figure 2. It is widely distributed in hotter parts of India, Ceylon, Saudi Arabia and tropical Africa from Mauritania and Senegal eastward to Eritrea, Ethiopia and Somalia and from there southward to South Africa and Madagascar. It occurs from sea-level up to 2100 meter altitude in grassland and bush land on many different soil types [40]. It is commonly known as "Tiny flower *Hibiscus*" in English [40] and "Nacha" in Amharic [41].

A B

Figure 2. Image of *Hibiscus micranthus* Linn. (A) plant (B) coarse dried powder

According to the research done by Ashoke *et.al*., (2011), the *n*-hexane extraction of the powder of dried leaves of *H. micranthus* by GC-MS analysis yielded major components of β-ionone,

carboxylic acid, oleic acid, hydrocarbons, aldehydes, ketones, sulphur compounds, fatty acids, esters and alcohols. From the root and stem extract, polyunsaturated fatty acids were exhibited which are important for growth, neural functions, as cardio-protective, anti-arthritis, anti-inflammatory and as anticancer [42]. The research which was done by Naji and Luca (2013), the highest anthocyanin content was obtained from methanol extract of *H.micranthus* than ethanol extract which may be responsible for antioxidant effects [43].

In India, the fruits and flowers of this plant is used as hypoglycemic agent. According to the secondary metabolites present in the plant, the plant has been used for its antipyretic, anti-inflammatory, hematological effects, antifungal, antiviral, antitumor, female antifertility, viralizing and anabolizing activities[44]. Some compounds like phenolic acids, tannins, flavonoids, β-sitosterol, alkanes, carbohydrates, steroids, fatty alcohols and acids have been reported [42, 45]. The roots are also used traditionally chewed as a cure for cough in India [46], used to cure venereal diseases in Sudan, applied as dressings on wounds and sores of humans and domestic animals and are also taken to cure bronchitis and pneumonia in Kenya and Tanzania [29]. In Tanzania, the leaves are used for treating earache, the leaf sap is taken against dysentery, water in which leaves have been pounded is taken against stomach-ache and leaf pulp is applied on swellings, used as an antidote for snakebites and as a treatment for kidney problems. In Tanzania and Zambia, the whole plant is used to treat convulsive fever in children [29].

In Ethiopia, the leaf of this plant provides medicines traditionally for treating skin burning (dermatological infections) and skeleto muscular disorders[43], swellings over body [47,48], for wound healing or sore in Tigray region [49]. The leaf and the flower of the plant is also used for wound and dermatological purposes by chewed and creamed with cotton in Amhara region, in and around Tara Gedam, South Gonder Zone [41]. *H. micranthus* also uses for burn in Negelle-Borona [50].

From literature search, no scientific investigations have been conducted till date to verify the use of *H.micranthus* as antimicrobial and wound healing remedies. The present study, therefore, focuses on delineation of the antibacterial activities of extracts of *H.micranthus* and its effects on wound by excision wound model.

1.6. Statement of the problem

As we know bacterial infections are one of the main problems in the world in general and should be treated by antimicrobial agents. The increased prevalence of known resistant organisms and the emergence of newly resistant organisms have resulted in delayed effective therapy and length of hospitalization and have led to increased cost for patients. Communicable diseases account for 50% of the disease burden of the developing countries, which represent 4.8 billion people, 80% of the world population [2].

Globally, nearly 340 infectious diseases are reported to have emerged between 1940 and 2004, including many drug-resistant strains of pathogens. The emerging infectious diseases also present danger to the health and security of the populations of the Organization for Economic Cooperation and Development (OECD) countries whether it is the rise of drug resistant pathogens, the threat of a global pandemic or the possibility of a bioterrorist attack [2, 51]. According to World Health Organization (WHO) and CDC, antibiotic resistance is now recognized as a global public health problem with major economic, social and political implications [52, 53]. More than 70% of the bacteria associated with hospital-acquired infections in the USA are resistant to one or more of the drugs previously used to treat them [5].

The impact of this problem is emphasized in low-resource countries because of the high prevalence of bacterial infections and the major role of antimicrobial agents in combating infectious diseases. Furthermore, the evolution of new strains of disease causing agents is of great concerns to the global health community. In addition to this problem, antimicrobials are sometimes associated with adverse effects on the host which include hypersensitivity, eradication of normal flora, immune suppression and allergic reaction.

Therefore, there is a need to look for compounds from other sources with confirmed antimicrobial and wound healing activity. This has led to the search for more effective antimicrobial agents among materials of plant origin with the aim of discovering potentially useful active ingredients that can serve as lead molecules and model for the synthesis of new antimicrobial drugs.

Although medicinal plants play a significant role in supporting the primary healthcare in Ethiopia, only a limited attempt has been done to scientifically explore [54]. A study of drug use in

2006 showed that 35% of the patients did not obtain the prescribed drugs due to lack of money. However, most traditional medicines are delivered either free or with a relatively low cost which contributes to the use of rural based healers for community primary health care need [55]. There is an increase interest to explore the secret of traditional herbal remedies based on information collected from local residents and traditional practitioners in different parts of the world. The search for new antibacterial agents has increased in the last decade mainly because of the increase in bacterial infections especially in countries with poor populations and more so because of bacterial resistance to current antibiotics [38].

Wound and wound infections also represent a major health problem both in terms of morbidity and mortality which are most common in developing countries because of poor hygienic conditions. Wound infection and associated delayed healing present considerable challenges for clinicians particularly with respect to identifying infection and choosing appropriate treatment options [12].

1.7. Significance of the study

The range of pathogenic bacteria is wide and a variety of diseases are caused by them. Despite the existence of potent antimicrobial agents, resistant or multi-resistant strains are continuously emerging imposing the need for a continuous search and development of new drugs. Hence; many efforts have been exploited to discover new antimicrobial compounds from various kinds of sources such as soil, microorganisms, animals and plants. One such resource is medicinal plants and their systematic screening may result in the discovery of novel effective compounds. In fact, plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines.

Medicinal plants used in traditional medicines offer a great reservoir for the discovery of new plants having antimicrobial properties comparable to antibiotics used in modern medicines. Since, almost all the antimicrobial agents are being imported and by considering the availability of medicinal plants in these countries, a lot of foreign exchange may be saved. In addition, the cost of treatment is steadily increasing and becoming unaffordable by common users. Therefore, development of therapeutic agents from our own indigenous resources will be of great help.

There is a constant need for effective and cost-effective therapies to promote wound healing and plant products offer an alternative that needs to be developed. Many plant materials including *H. micranthus* have been used topically and systemically to enhance wound repair though evidence is lacking. There is, however, a need to study and provide evidence for the efficacy of *H. micranthus* extract in the treatment of wounds.

The results of pharmacological screening of *H. micranthus* will have a contribution in the discovery of various medicinal plants with antibacterial and wound healing activity. This will also provide a base line data for further investigations on this plant.

2. OBJECTIVES

2.1. General objective

- To investigate antibacterial and wound healing activity of the methanol leaves extract of *Hibiscus micranthus* Linn.

2.2. Specific objectives

- To perform phytochemical screening of the methanol leaves extract of *H. micranthus*
- To evaluate the acute oral toxicity profile of the methanol leaves extract of *H. micranthus*
- To evaluate the skin sensitivity to the methanol leaves extract of *H. micranthus*
- To evaluate the antibacterial activity of the methanol extract of *H. micranthus* leaves against standard strains
- To investigate the wound healing activity of the methanol extract of the leaves of *H. micranthus.*

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Ampicillin, ciprofloxacin, methanol (RFCL limited, New Delhi, India), distilled water, nutrient broth (India), blood agar, MacConkey agar, Mueller Hinton Agar, biochemical testing, DMSO (dimethyl sulphoxide- Loba Chemie Pvt.Ltd. Mumbai, India), phytochemical screening reagents, ketamine(ROTEXMEDICA, Germany), simple ointment base and nitrofurazone ointment USP 0.2% (Galentic pharma, India Pvt Ltd.)were purchased and collected from the respective sources.

3.2. Instruments and medical supplies

Oven, glass test tubes, scissors, petridish, beaker, bottle, filter paper, surgical blade, conical flasks, centrifuge, refrigerator, cotton, shaker, incubator, analytical balance, bunsen burner, micropipette, glass pipette, graduated cylinders, rulers, wire loops, sterile swab, sticker, oral gavages, disposable syringes, gown and glove.

3.3. Plant material collection and identification

Enough amounts of the fresh leaves of *H.micranthus* were collected from Gondar area, Ethiopia during the Month of January 2015.The plant was identified by a Taxonomist(Ato Melaku Wondaferash) and a voucher specimen representing *H.micranthus* (Specimen No. AA 004) was deposited at the National Herbarium, Department of Biology, Addis Ababa University, Ethiopia.

3.4. Test organisms

In the present study, the following microorganisms obtained from the American Type Culture Collection (ATCC) were used to determine the antibacterial activity of *H.micranthus*. The gram positive bacterial strains used were *S. aureus*(ATCC2923), *S.pneumoniae*(ATCC137348) and *S. pyogenes*(ATCC19615)while gram negative bacteria strains used were *E.coli* (ATCC1925525), *P.aeruginosa* (ATCC27853), *K.pneumoniae*(ATCC70060) and *P. mirabilis*(ATCC12386).The bacterial strains were obtained from University of Gondar Referral Teaching Hospital Laboratory and Microbiology Department. Morphological characteristics (shape, size, form, opacity and pigment production turbidity) of the 24 hours bacterial cultures were observed. Pure culture of bacteria was maintained at 4^{o}c on nutrient agar slants. The identity of these bacterial isolates was confirmed through the conventional biochemical tests [9, 27].

3.5. Experimental animals

Healthy adult Wistar albino rats of either sex were selected randomly for the study. Rats were obtained from animal breeding house of School of Pharmacy, University of Gondar. Rats of 12 weeks, weighing 160-220 g were used for experiment. Each rat was housed in plastic box cage under controlled conditions at 19-25^{0}c and kept under 12/12 light/dark cycle with free access to standard pellet feed brought from Ethiopian Health and Nutrition Research Institute, Addis Ababa and water *ad libitum.* They were all acclimatized to the Pharmacology Laboratory prior to use. The study was carried out according to the National Research Council Guide for the Care and Use of Laboratory Animals and OECD guide line [56, 57] after obtaining the approval of the Institutional Animal Ethical Committee (IAEC). The minimum number of animals and duration of observation required to obtain consistent data was employed.

3.6. Experimental design

The study utilized an *in vitro* antibacterial and *in vivo* experimental study for acute oral and dermal toxicity test and for wound healing activity test. Crude extract of the plant leaves was prepared with maceration. The extract was then screened for phytochemical, acute oral and dermal toxicity tests, antibacterial and wound healing activity tests by their respective methods.

3.6.1. Methods

3.6.1.1. Extraction procedure

Fresh matured leaves of *H.micranrhus* was collected, washed, dried under shade and extracted as described by Kumar *et al.*,(2010) [45] with slight modification. The dried leaves were coarsely powdered using a mortar. Then, this coarsely powdered plant was macerated in 80 % methanol to obtain the hydroalcoholic crude extract using Erlenmeyer flask for 3 days at room temperature. After 72 hours, the filtrate was separated from the marc by using filter paper (Whatman No.1). The marc was re-macerated twice. Then the alcohol was allowed to evaporate from the filtrate with mild heating on dry oven to dry at 40^{o}c for how long and then the concentrated extract was stored at 4^{o}c until the actual experiment is done.

3.6.1.2. Preliminary phytochemical screening

The crude methanol extract was assessed for secondary metabolites such as phenol, tannin, flavonoids, saponins, terpenoids, triterpenes, diterpines, glycosides, anthraquinones, alkaloids and steroids using standard methods [19, 32, 45] as shown in Table 1 below.

Table1. Phytochemical screening of *Hibiscus micranthus* leaves

Tests		Standard inference	
	Reagents to be used	**Color inference**	**Color indication**
Phenol	10% ferric chloride + distilled water + extract	green/greenish yellow	presence of phenols
Tannin	10% ferric chloride + 2 ml diluted sample	brownish green/ greenish black	presence of tannins
Flavonoids	5 ml of dilute NH_3 + 3 ml extract + 2 ml of conc. H_2SO_4	Yellow	presence of flavonoids
Saponins	0.5gm of extract + 10 ml distilled H_2O + shake for some minutes	frothing/formation of foam	presence of saponins
Terpenoids	5ml of extract + 2ml chloroform + 3 ml conc. H_2SO_4	reddish brown	presence of terpenoids
Triterpines	Extract +chloroform then filtrate +conc.H_2SO_4	golden yellow	presence of triterpines
Diterpines	Extract +Distilled water + 3-4 drops of copper acetate	emerald green	presence of diterpines
Glycosides	5 ml of extract+ 2 ml glacial acetic acid + 1 ml conc. H_2SO_4 +1 drop $FeCl_3$	brown ring	presence of glycosides
Anthraquinones	5.0 g of extract + 10 ml of benzene + 5.0 ml of 10% ammonia to the filtrate.	violet color	presence of anthraquinones
Alkaloids	2 ml extract + Mayer's reagent	reddish brown	presence of alkaloids
Steroids	1 ml of the extract +10ml of chloroform + conc. H_2SO_4	the upper layer turns red & H_2SO_4 layer showed yellow with green flourecence	presence of steroids

3.6.1.3. Acute oral toxicity test

Acute toxicity study was carried out using the limit test dose of 2g/kg as described by OECD (2008) guideline and Interagency Research Animal Committee Recommendation [56, 57]. Five animals of either sex were fasted overnight but water was allowed and was administered with the limit dose 2g/kg of *H.micranthus* extract. Animals were observed individually for behavioral profile (alertness, restlessness, irritability and fearfulness), autonomic profiles (defecation and urination), neurologic profile (spontaneous activity, reactivity, touch response, pain response and gait), physical states as lacrimation, loss of appetite, tremors, hair erection, salivation, diarrhea and for mortality after dosing at attentively during the first 30 minutes, with special attention given during the first 4 h, periodically during the first 24 h and daily thereafter for a total of 14 days.

3.6.1.4. Acute dermal toxicity test

The skin irritation test with 80% methanol extract of *Hibiscus micranthus* was conducted on rats. Animals showing normal skin texture were housed in a cage. Five rats were employed for this test and their skin was shaved on the dorsal side, each about 400 mm^2 areas 24 h before application of the sample. A limit test dose of 2000 mg/kg of formulation was applied uniformly over the shaved area for 24 h. The plant extract was held in contact with the skin using a zinc oxide adhesive plaster and a non-occlusive bandage dressing for 24 h, at the end of the exposure period, the bandage and the test materials were removed and observed for an adverse skin reaction/irritation. Then, they were further observed for 14 days for any signs of toxicity [58, 59, 60].

3.6.1.5. Methodology for detection of antibacterial activity

3.6.1.5.1. Inoculums preparation

The bacterial isolates were first grown in a nutrient broth for 18 h before use. The microbial inoculums were standardized by 0.5 McFarland. Inoculums preparation of 0.5 McFarland turbidity standards was done by using a sterile inoculating loop touch isolated colonies of the same morphology were suspended in 5 ml of nutrient broth in to sterile test tubes for the identified bacteria. 0.5 McFarland standards were vigorously agitated to turbidity on a vortex mixer before use. As with the barium sulfate standards, a 0.5 McFarland standard was comparable to a bacterial suspension of 10^8 cells per /ml. The standard was compared visually to a suspension of bacteria in nutrient broth accordingly [9].

3.6.1.5.2. Antibacterial assay

The antibacterial activity of methanolic extracts of *H. micranthus* leaves was tested against clinical isolates by agar-well diffusion method as described by Sohel *et al*., and Bahur *et al.*, [27, 61]. A Cork borer of 6 mm diameter was used to punch well in agar plates to cut uniform wells for studying antibacterial activity *in vitro* by agar-well diffusion method [27]. Then, an aliquot of 100 µl inocula (McFarland turbidity standard) for each bacterial isolate was evenly spread by a sterile glass spreader onto a previously bored on to Muller Hinton Agar using sterilized cotton swab and was allowed at room temperature. Different concentrations of the extract (200, 400 and 800 µg/ml) were prepared using DMSO as solvent. Subsequently, 200, 400 and 800 µg/ml extracts of leaves were poured into the wells. Approximately 0.1 ml of stock solution prepared with DMSO was introduced into the well and allowed to stand at room temperature and then was incubated at 37°C as positive control for bacteria. Ciprofloxacin 5µg and ampicillin 10µg discs were used as positive control. DMSO was used as a negative control. Then the plates were kept at 2-8^{o}c in a refrigerator to allow diffusion of the extracts in to the agar and further incubated at 37^{o}c for 24 hours. The diameter of zone of inhibition was measured to the nearest millimeter [27, 62]. The formation of clear inhibition zone of $\geq$7 mm diameters around the wells was regarded as significant susceptibility of the organisms to the extract [63]. The effect was compared to those of antibiotic discs. The tests were performed in duplicates and the mean was taken. The whole experiments were performed under strict aseptic conditions.

3.6.1.5.3. Determination of Minimum Inhibitory Concentration (MIC)

The MIC test was performed for bacteria species susceptible by disc diffusion test and the estimation of MICs of the extraction was investigated per the Clinical Laboratory Standards Institute [64]. MIC was conducted using macro-tube serial dilution methods. The extract (stock) solution was prepared and serially dilutions were done in DMSO. The extract solutions were prepared in different test tubes. The extract solution (5 mg/ml) was serially diluted as 1:1, 1:2, 1:4, 1:8 and 1:16 to bring 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625mg/ml and 0.3125mg/ml concentrations respectively. Varying concentration of the extract (5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml and 0.3125mg/ml) in test tubes containing 10 ml nutrient broth was added and then a loopful of the test organisms was introduced in each test tube. All inoculated dilutions were incubated at 37°c for 24 hours. The lowest concentration of the plant extract that

retains its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism was recorded as the MIC value of the extract.

3.6.1.5.4. Determination of Minimum Bactericidal Concentration (MBC)

Determination of MBC of the extract was also investigated by the method described by the Clinical Laboratory Standards Institute [64]. A loopful sample was then taken from each set of test tubes with no visible growth in MIC assay and sub-cultured on freshly prepared Nutrient agar plate and later incubated at 37°C for 24 h. After incubation, the lowest concentration of the plant extracts showing no bacteria growth was recorded as MBC.

3.6.1.6. Grouping and dosing of animals

The animals were randomly assigned to four groups of each consisting of six rats each. The animals of Group I was used for simple ointment base, Group II was served as reference standard and treated with 0.2% w/w nitrofurazone ointment and animals of Group III and IV were treated with ointments prepared from 5% and 10% concentrations of methanolic extract of *H. micranthus* respectively. Dose of ketamine anaesthesia (50mg/kg, ip) for wounding procedure was selected based on previous studies120mg/kg, ip [65], 30mg/kg ,ip[66] and 50mg/kg sc[59].The test doses were prepared freshly on the day of the experiment.

3.6.1.7. Methods for wound healing activity

3.6.1.7.1. Ointment formulation

Two types of ointment formulations were prepared from the extract. Five percent (w/w) and 10% (w/w), where 5 or 10g of the extract were incorporated in to 100 g of simple ointment base respectively [67,68]. Nitrofurazone ointment (0.2% w/w) was used as a standard drug for comparing the wound healing potential of the extract [65, 68].

3.6.1.7.2. Methods for creating excision wound

The animals were anesthetized with ketamine 50mg/kg body weight. The rats were inflicted with excision wounds as described by Mekonnen *et al*., and Morton and Malone [59, 69] as shown in Figure 3. The dorsal fur of the animals was shaved with small scissors and the anticipated area of the wound to be created was outlined on the back of the animal with marker. The excision wound was made by cutting away a circular area 210 - 240 mm^2 and 1-2 mm depth full thickness of skin from the depilated area along the marking using toothed forceps, a surgical blade and pointed scissors. The wound was left undressed to open environment [59, 65].

3.6.1.7.3. Treatment methods

The ointment was topically applied once a day starting from the day of the operation till complete epithelization. This model was used to monitor wound contraction and wound closure time. Wound contraction was calculated as percentage reduction in wound area. The progressive changes in wound area were monitored planimetrically by measuring the diameter every alternate day. The period of epithelialization was calculated as the number of days required for falling of the dead tissue remnants of the wound without any residual raw wound [65]. The wound area was measured on alternate days and the epithelialization period recorded at the end of the study. Wound contraction (%) was calculated using the relation [28, 70]:

Wound contraction (%) = $[(W_{A0}-W_{At})/W_{A0}]$ 100

Where:

W_{A0} = the wound area on day zero

W_{At} = the wound area on day t.

3.7. Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with post hoc Tukey's Multiple range test with SPSS version-16 for windows. $P < 0.05$ was considered significant and all data was expressed as mean ± SEM.

3.8. Quality control and assurance

The quality assurance of the experimental study was taken care of according to the standard working principle using quality assuring physical and chemical instruments and methods in collaboration of Department of Pharmacognosy, Pharmacology and Microbiology. Testing of MIC and MBC were done based on National Culture Collection Laboratory Standard (NCCLS) [9]. The quality of solvents extraction, number of extraction and time of extraction were kept according to the standards. Drying and concentration procedures were also ensured to keep the safety and stability of the active constituents. The quality of the data was also controlled using computerized method.

3.9. Ethical considerations

Animal handling was done according to the guide lines for Care and Use of Laboratory Animals [56] and OECD-guidelines-425 [57]. The proposal was submitted to Department of Pharmacology for approval and then the study was undertaken after obtaining the approval of IAEC of University of Gondar (IAEC approval letter No. SoP4/407/2015 dated 28th April 2015). The certificate of clearance was obtained from Department of Pharmacology to procede the research.

4. RESULTS

4.1. Yield of the plant's extract

The *Hibiscus micranthus* leaves were extracted for the crude active extracts of 80% methanol. From 100g of *H. micranthus* leaves extract, 11.6%w/w was extracted using methanol. The extract was greenish black.

4.2. Phytochemical screenenig

Chemical analysis of the powdered plant was carried out qualitatively to associate the antibacterial and wound healing activities of the plant extract. Preliminary phytochemical screening of the leaves extract of *H. micranthus* revealed the presence of alkaloids, flavonoids, saponins, tannins, steroids, phenols, diterpines, anthraquinones and the absence of terpenoids, glycosides and triterpines (Table 2).

Table 2.Phytochemical profile of methanol extract of *Hibiscus micranthus* leaves

Chemical constituents	Inference
Alkaloids	+
Flavonoids	+
Saponins	+
Tannins	+
Steroids	+
Phenols	+
Diterpines	+
Anthraquinones	+
Terpenoids	-
Glycosides	-
Triterpines	-

+ = present, - = absent

4.3. Antibacterial activity of plant extracts using Agar well diffusion

The extract showed antibacterial activity as indicated by the zone of growth inhibition ranged from 14±.000-22.67±1.202mm. *S. aureus (*which show significant difference with the positive control ampicillin at *p=0.021* and *S. pneumoniae* strain in a concentration dependent fashion (which shows significant difference with the positive control ampicillin at *p=0.003* and ciprofloxacin at *p=0.016)* had the largest zone of inhibition (22.67±1.202 mm) at concentration of 800µg/ml and 400µg/ml respectively followed by *P.aeuruginosa* [(which shows significant difference with the positive control ampicillin at *p=0.037* and ciprofloxacin at *p<0.001)* (18.33±.333mm)] and *K.pneumoniae* [(which shows significant difference with the positive control ampicillin at *p=0.040* (16.33±1.202mm)] at a concentration of 400µg/ml and 800µg/ml respectively while *S. pyogenes* had the smallest zone of inhibition (14±.000mm) at a concentration of 400µg/ml. However, *E.coli* and *P.mirabilis* were found to be resistant (.00±.000 mm) to the leaves extract at any of the applied doses (Table 3).

Table 3.Diameter of zone of inhibition (mm) against bacteria by *H.micranthus* leaves extract

	Conc.			Mean zone of inhibition(mm) ± SEM			
Bacterial strain	µg/ml	p-value	Between	Extract	Cipr 5µg	Ampi 10µg	DMSO100µl
S. aureus	200	0.021	Extract-ampi	18.33±1.667			
	400	-	-	20.00±1.555	21.67±1.202	25.33±1.667	.00±.000
	800	-	-	22.67±1.202			
S. pneumoniae	200	-	-	20.67±1.764			
	400	-	-	22.67±1.202	19.33±.333	21.00±.577	.00±.000
	800	0.016	Extract-cipr	13.00±1.528			
		0.03	Extract-ampi				
S. pyogenes	200	-	-	.00±.000			
	400	-	-	14±.000	23.33±.667	27.00±.577	.00±.000
	800	-	-	.00±.000			
P.aeuruginosa	200	<0.001	Extract-cipr	.00±.000			
	400	0.037	Extract-ampi	18.33±.333	27.00±.577	6.00±6.00	.00±.000
	800	<0.001	Extract-cipr	.00±.000			
K.pneumoniae	200	0.013	Extract-ampi	13.67±1.856			
	400	0.040	Extract-ampi	14.67±.667	18.00±1.453	20.00±.577	.00±.000
	800	-	-	16.33±1.202			
E.coli	200	-	-	.00±.000			
	400	-	-	.00±.000	23.67±1.453	19.33±.333	.00±.000
	800	-	-	.00±.000			
P.mirabilis	200	-	-	.00±.000			
	400	-	-	.00±.000	30.00±.000	26.33±.333	.00±.000
	800	-	-	.00±.000			

Conc- concentration of the extract; Cipr-ciprofloxacin; Ampi-ampicillin; DMSO–Dimethyl sulphoxide, mm-millimeter, µg-microgram, µl-microlitre; ml-mililitre; SEM-standard error of the mean; '0' - no zone of inhibition;(-)- no significant difference between extract and standard drugs. P-values <0.05 indicates significant difference between the extract and the standard drugs or positive controls. Values greater than 6mm diameter of the well indicate some activity.

4.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC were tested for the methanol extract of *H.micranthus* leaves and the results are presented in Table 4. *S. pneumoniae* had the lowest MIC value (0.625mg/ml), while *S. aureus* and *P.aeuruginosa* had MIC value of 2.5mg/ml and 1.25mg/ml respectively. *K.pneumoniae* had the highest MIC value of 5mg/ml. Higher MBC of 5mg/ml was observed against *S. aureus*, *P.aeuruginosa* and *K.pneumoniae.* MBC value of 1.25 mg/ml against *S.pneumoniae* was reported (Table 4).

Table 4.MIC and MBC of *H.micranthus* leaves extract against test organisms

Bacterial strain	MIC(mg/ml)	MBC(mg/ml)
S. aureus	2.5	5
S. pneumonia	0.625	1.25
S. pyogenes	ND	ND
P.aeuruginosa	1.25	5
K.pneumoniae	5	5
E.coli	ND	ND
P.mirabilis	ND	ND

ND- Not Determined; MIC- Minimum Inhibitory Concentration; MBC- Minimum Bactericidal Concentration; mg/ml - milligram per milliliter

4.5. Results of acute oral toxicity

All the rats which received *H.micranthus* at the dose up to 2 g/kg body weight did not result in signs of toxicity or mortality. The animals were physically active, consuming food and water as regular. Any sign of abnormal behavior has not been noticed. Based on the result of acute toxicity, $LD_{50\%}$ was estimated to be above 2gm/ kg body weight.

4.6. Results of acute dermal toxicity

The purpose of this study was to assess the skin irritation potential of *Hibiscus micranthus* from a single topical application. During the entire period of experimentation, no animal showed a sign of abnormal behavior.

4.7. Wound healing effect of *Hibiscus micranthus* in excision wound model

The measurements of the progress of the wound healing include by the NFZ ointment (0.2%w/w), extract ointment 5 and 10% w/w and the control group (simple ointment base) in the excision wound model are shown in Table 5. The two doses of the plant extract 5 and 10% w/w exhibited significant wound contraction rate as compared to NFZ ointment and simple ointment base treated group from 6^{th} to 16^{th} day respectively ($p<0.05$). It was found that the wound healing contracting ability of the extract ointment in different concentrations was significantly greater ($p<0.05$) than that of the control (i.e. simple ointment treated group) as well as reference standard (NFZ ointment) starting from the sixth day onwards. The extract ointment produced complete healing at the 16^{th} day when 5 and 10 % w/w extract ointments were used. The extract treated wounds were found to epitalize faster compared to the control group.

Table 5.Effect of the methanol extract of *H.micranthus* leaves on excision wound model (n=6)

Post wounding days	Wound surface area (mean ± SEM) in mm^2 and percentage of wound contraction			
	Ointment base	Nitrofurazone ointment (0.2%w/w)	Extract ointment (5% w/w each)	Extract ointment (10% w/w each)
0	220.60±2.713(0.0)	213.80±7.971(0.0)	227.80±5.643(0.0)	230.80±2.871(0.0)
2	180.80±4.769(18.04)	161.20±7.010(24.60)	166.00±9.508(27.13)	167.80±12.395(27.30)
4	145.40±7.096(34.09)	136.00±6.782(36.39)	119.20±10.851(47.67)	117.40±9.765(49.13)
6	113.80±5.643(48.41)	80.00±8.390(62.58)	76.20±8.777*(66.55)	83.40±10.028(63.86)
8	70.80±11.128(67.91)	44.40±4.261(79.23)	36.60±5.600*(83.93)	53.60±7.467(76.78)
10	34.60±4.707(84.32)	20.60±2.088(90.36)	16.80±1.497*(92.63)	29.40±5.400(87.26)
12	19.80±1.428(91.02)	14.40±2.400(93.26)	10.80±1.200*(95.26)	11.60±2.400*(94.97)
14	9.40±1.166(95.74)	7.20±3.200(96.63)	2.40±0.400(98.95)	5.20±1.068(97.75)
16	6.60±0.872(97.00)	3.80±2.835(98.22)	1.60±1.666(99.30)	2.00±1.095(99.13)

Values in parenthesis indicate percentage of wound contraction.* significant differences at $p<0.05$ (statistical analysis was done by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons).

5. DISCUSSION

Now a day, medicinal plants play a major role in the treatment of infectious disease [22-27]. The WHO estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the words population. Ethnobotanical investigations have been found to recommend main clues in the discovery and development of traditionally used medicinal plants into modern drugs [33]. The medicinal values of these plants lie in bioactive phytochemical constituents that produce definite physiological function on the human body and those compounds formed are the foundations of modern prescription drugs as we know today[32].

Antibiotic resistance is a major concern and development of new agents from plants could be useful in meeting the demand for new antimicrobial agents with improved safety and efficacy. Newer antimicrobial agents from plant extracts may be useful in food, dairy and pharmaceutical industries to prevent contamination by limiting the microbial growth [71]. The leaves extract of *H.micranthus* demonstrated significant antibacterial activity against most of the test organisms. The extract was more potent against gram-positive *S. aureus* and *S.pneumoniae* with maximum zone of growth inhibition of 22.67±1.202mm at 800µg/ml and 400µg/ml respectively. Though, *S. pyogenes* had the smallest zone of inhibition (14±.000mm) compared with other gram positive bacteria at a concentration of 400µg/ml, it was found that the extract had an inhibitory effect on this strain.

The plant extract inhibited gram-positive microorganisms better than the gram-negative ones. This is in agreement with previous reports [72]. Although gram-negative bacteria tend to have higher intrinsic resistance to most antimicrobial agents [73], impressive activity against some gram negative bacteria was observed. Low values of MIC (0.625 mg/ml) and MBC (1.25mg/ml) demonstrated by the extract especially on *S.pneumonia*e is an indication that the phytoconstituents have bactericidal potential. Similarly, the MIC (1.25 mg/ml) and MBC (5 mg/ml) exhibited against *P.aeuruginosa.* The inhibitory effects of the extract of *H.micranthus* leaves against pathogenic bacterial strains can introduce the plant as potential candidate for drug development for treatment of ailments caused by these pathogens.

The results of the antibacterial effect of *H.micranthus* on *E.coli* and *P.mirabilis* indicate that this bacterium presents a resistance even in higher concentration the fact that the diameter of the inhibition zone was zero in all petridishes inoculated by these strains. While the antibacterial

activities of the methanol extract of *H.micranthus* leaves compared to standard antibiotics (ciprofloxacin and ampicillin) showed broad spectrum as its activity were independent on gram reaction. The extract tested showed antimicrobial activity against both gram positive (*S.aureus, S.pneumoniae* and *S.pyogenes*) and gram negative organisms (*P. aeruginosa* and *K.pneumoniae*). The Present investigation confirms, therefore, the antibacterial activity of extracts of *H.micranthus* leaves. These activities are supported by the presence of high level of alkaloids, terpenoids, diterpines, tannins, saponins, phenols, flavonoids, glycosides and steroids which might be responsible for the antibacterial activities [32, 73]. Anthraquinone also has antibacterial effect as described by Marjorie Murphy Cowan (1999) [74].

In the phytochemical screening of this study, the methanol extract of *H.micranthus* showed positive indication for the presence of alkaloids, flavonoids, saponins, tannins, steroids, phenols, diterpines and anthraquinones. Therefore, the observed antibacterial activity of the methanol extract can be attributed primarily by the nature of biologically active components of the plant like alkaloids, terpenoids, diterpines, tannins, saponins, phenols, flavonoids, glycosides and steroids which are well known for their antimicrobial activity [32, 71] supporting the traditional use of the plant and secondarily by the stronger extraction capacity of methanol could have produced a large number of active constituents responsible for antibacterial activity [26,43]. Expected mode of antimicrobial action of some secondary metabolites may be related for example tannins- inactivate microbial adhesins, enzymes, cell envelope transport proteins, flavonoids- microbial target is the membrane, alkaloids- intercalate in to cell wall and /or DNA, diterpines and phenolic substances- membrane disruption)[74]. These secondary compounds may come into play (either individually or synergistically) to confer the antibacterial potential of this plant.

From the literature, the n-hexane extract of the *H. micranthus* leaves contains carboxylic acid, oleic acid, hydrocarbons, aldehydes, ketones, sulphur compounds, fatty acids, esters and alcohols. Presence of aldehydes has been used for perfuming agent, flavoring agent, antiseptic, insect repellant and aromatic compounds and also phenolics like eugenol usually posses antioxidant, antimicrobial, antifungal and used as several other therapeutic uses. Presence of fatty acids is important for growth, neural functions, as cardio-protective, anti-arthritis, anti-inflammatory and as anticancer [42]. The highest anthocyanin content of methanol extract of *H.micranthus* may be responsible for antioxidant effects [43]. According to the secondary

metabolites present in fruits and flowers of the plant, the plant has been used for its antipyretic, anti-inflammatory, hematological effects, antifungal, antiviral, antitumor, female anti-fertility, viralizing, hypoglycemic agent and anabolizing activities [44].

H.micranthus leaves have been extensively used for treating earache, dysentery, stomach-ache, applied on swellings, as an antidote for snakebites and as a treatment for kidney problems. The whole plant is used to treat fever, especially convulsive fever in children by Tanzanian and Zambian people [29]. In Ethiopia, the leaf of this plant is traditionally used to treat skin burning (dermatological infections) [43, 41, 50] and skeleto muscular disorders [43], swellings over body [47, 48], for wound healing and sore [49, 41]. The flower of the plant is used for wound and dermatological disorders traditionally [41]. The root preparations are used to cure venereal diseases in Sudan and are also applied as dressings on wounds and sores of humans and domestic animals and are also taken to cure bronchitis and pneumoniae in Kenya and Tanzania [29]. These were in support of the current study in having antibacterial and wound healing effect. Findings of the present study clearly demonstrate the scientific basis of traditional medication with the extracts prepared from *H. micranthus* and reveals its potential use as complementary and alternative medicine.

Wound healing is an orderly progression of events that establish the integrity of the tissues. Wound healing process begins with restoration of damaged tissue as closely as possible to its natural state and wound contraction is the course of shrinkage in wounded area [75]. In the present study, *H.micranthus* leaves extract showed increased rate of wound contraction and epithelialization. Topical application of the extract on excision wound accelerated wound contraction and reduced epithelialization period in rats. Thus, the effect of the extract on wound contraction and epithelialization suggests, it may enhance epithelial cells migration and proliferation as well as the formation, migration and action of myofibroblasts. It is, therefore, likely that in addition to enhancing wound contraction and epithelialization, the extract may also stimulate processes associated with tissue regeneration. The significant responses to the extract reported above may be attributed to properties that include antioxidant, anticonvulsant, and hypoglycemic activities [65].

A variety of *in vitro* and *in vivo* experiments have shown that some plants metabolites such as tannins/phenolic compounds, flavonoids, and terpenoids promote wound healing process mainly

due to their astringent and antimicrobial property which seems to be responsible for wound contraction and increased rate of epithelialization [76]. Flavonoids, tannins and simple phenolic compounds possess anti-inflammatory and antioxidant properties and could contribute to the wound healing properties of *H.micranthus*. Flavonoids are known to reduce lipid per oxidation by preventing or slowing onset of cell necrosis and improving vascularity, hence increasing the strength of collagen fibers by increasing circulation or by preventing cell damage and by promoting DNA synthesis [76]. Previous studies also showed that the chemical constituents of *H.micranthus* which included the anthocyanin which may be responsible for antioxidant effects [43]. The extract showed positive indication for the presence of alkaloids, flavonoids, saponins, tannins, phenols, diterpines and anthraquinones which are responsible and supported the wound healing activity. These bioactive agents usually modulate one or more phases of the healing process and are multifunctional natural products that act through multiple targets by being anti-inflammatory, antioxidant, etc.

For example, the anti-inflammatory activity of flavonoids has been shown to be attributed to their ability to inhibit neutrophil degranulation; diminishing the release of arachidonic acid and other mediators from immune cells. Alkaloids promotes early phases of wound healing ($\leq$ 7 days), stimulate the growth of colonies from fibroblast precursors. On the other hand, saponins have been shown to modulate wound cells function, can increase both fibroblast proliferation and migration, showing greater cell density, more regularly organized dermis and more newly formed blood vessels. The anthraquinone has been shown to promote excisional wound repair in rats via complex mechanism involving stimulation of tissue regeneration. Tannins typically act as astringents and are found in a variety of herbal products used for wound healing. This astringent property is responsible for wound contraction and increased rate of epithelialization at the granulation formation and scar remolding phases. Phenolic compounds have been documented to possess potent antioxidant and free radical scavenging effect, which is believed to be one of the most important components of wound healing. The wound healing effect of tannins may also be attributed to their anti-inflammatory activity due to their antioxidant action [77]. Sulfur containing compounds are also important in healing accelerated efficacy and antimicrobial potency [68]. The extract has been reported to have a broad spectrum antibacterial activity in this study, which also seemed to have beneficial effects on promoting wound healing.

The result showed that the wound area recorded for each extract-ointment decreased as the days of exposure increased, while the respective percentage wound contraction increased with increase in the number of days of exposure to the extract ointments. It was observed that the wound healing contracting ability of the extract ointment in different concentrations was significantly greater ($p<0.05$) than that of the control (i.e. simple ointment treated group) as well as reference standard (NFZ ointment). The 5% (w/w) extract ointment treated groups showed significant wound healing ($p<0.05$) from the 6^{th} day onwards where as the 10% (w/w) extract ointment treated group showed significant wound healing ($p<0.05$) from the 12^{th} day. The percentage of wound contraction was somewhat much more with the 5% w/w extract ointment treated group (1.60 ± 1.666 mm^2) than the 10% w/w extract ointment treated group ($2.00\pm1.095mm^2$) and the NFZ ointment (3.80 ± 2.833 mm^2) area on the 16^{th} day respectively. The wound healing activity of the plant extract may also be due to its angiogenic and mitogenic potential leading to increased cellular proliferation and increased collagen synthesis. Collagen gives strength and integrity to the tissue matrix and plays a role in homeostasis and epitalization at the latter phase of healing [68]. From clinical studies, it is shown that terpenoids strengthen the skin, increase the concentration of antioxidants in wounds and restore inflamed tissue by increasing blood supply. Because of these properties, *H.micranthus* has been used widely in herbal medicines for burns, psoriasis and prevention of scar formation following surgery [32].

It appears that *H.micranthus* has prohealing effect as evidenced by the above findings and was able to promote epithelialization either by facilitating the proliferation of epithelial cells or by increasing the viability of epithelial cells. Due to the various properties as discussed above *H.micranthus* could be used to treat open wounds. However confirmation of this conclusion requires clinical evaluation.

6. LIMITATION OF THE STUDY

This research work was not completed without any challenges and bypassed in collaboration of scores of people and different Departments of College of Medicine and Health Sciences of the University of Gondar, but the following limitations had been faced during the experimental work lack of sufficient chemicals around in the study area and lack of instruments for further study.

7. CONCLUSION AND RECOMMENDATIONS

7.1. Conclusion

H.micranthus is one of the medicinal plants used traditionally for various ailments in different countries including Ethiopia. The present study suggests that the methanol extract of the leaves exhibited a potential antibacterial activity against the tested microoragnisms which may be attributed to the presence of phytoconstituents like alkaloids, flavonoids, saponins, tannins, steroids, phenols, anthraquinones and diterpines and wound healing property which may be attributed to the presence of phytoconstituents like alkaloids, flavonoids, saponins, tannins, phenols, diterpines and anthraquinones to the extract. The extract inhibited both gram positive and gram negative organisms used confirming them to have broad spectrum. The antibacterial activity, enhanced wound contraction and shortened epithelialization period support the wound healing promoting effect of *H.micranthus*. However, identification and elucidation of the active constituents in this plant may provide useful leads to the development of new and effective drugs against different types of wounds and various infectious diseases. The results obtained from this study support the traditional claim of the plant for their use against earache, wound, sore throat, dysentery, burn or dermatological infections and other many infections. The extract was safe and shows no sign of toxicity or mortality with a dose of 2 g/kg body weight.

7.2. Recommendations

Based on this study, the following recommendations can be made:

- Further studies with isolated constituents compared to the crude extracts might be needed to comprehend the complete mechanism of antibacterial and wound healing activity and toxicological study of *H.micranthus*.
- Future studies may include a greater range of organisms including drug resistance clinically isolated bacteria.
- Simultaneous study of herbal along with synthetic antibacterial drug should be done

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9. ANNEXS

Annex 1 - Media preparation

A. Nutrient agar

Agar----------15.0g

Peptone----------5.0g

NaCl----------5.0g

Yeast extract----------2.0g

Beef extract----------1.0g

Distilled water----------1000ml

PH 7.4±0.2 at 25^{o}C

Source; this medium is available as a premixed powder from Oxoid Unipath Preparation of medium; add components to distilled water and bring volume to 1.0L. Mix thoroughly. Gently heat and bring to boiling. Distribute in to tubes or flasks. Autoclave for 15 minutes at pressure of 15psi and a temperature of 121^{o}C. **Use;** used for the cultivation and maintenance of a wide variety of microorganisms.

B. Nutrient broth

Peptone ……5.0g

NaCl…….5.0g

Yeast extract…….2.0g

Beef extract……..1.0g

Distilled water……..1000ml

C. Muller Hinton media

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. This formula conforms to Clinical and Laboratory Standard Institute (CLSI).

Formula / Liter

Beef Extract.....2 g

Acid Hydrolysate of Casein.....17.5 g

Starch.....1.5 g

Agar.....17 g

Final pH 7.3 ± 0.1 at 25°C

Formula may be adjusted and/or supplemented as required to meet performance specifications.

Annex 2- Sterilizing of Materials

Principle

All pathogenic and major contaminant including spores that were interfering our work would be eliminated by heat under pressure (Autoclave).

Procedure that will be used during sterilization

a) Put the used plates and tubes in the steel bucket
b) Add and check the level of water inside the autoclave until three markers are filled completely.
c) Insert the bucket which contain used items inside the autoclave
d) Cap the lid of the autoclave by tightening the handle nut.
e) Turn the black nodule (valve) up to number 6 times and the inside temperature of the autoclave will be displayed on the digital screen at middle height.
f) Wait until the existent temperature of the autoclave is stabilized.
g) Gave time of 30 minutes, the first 15 minute for stabilizing internal pressure and the second 15 minutes to sterilize completely at 121^0c with a pressure of 15pa and the second front valve will maintain the pure internal pressure maintained at 15pa.
h) Wait until it sterilize and cool down to less than 5^0 C
i) Open the lid and take out the sterilized items from the autoclave

Important precautions;

- The level of water before started will be cheeked
- The lid will be secured before started
- will not be tried to open when hot

Annex 3- Sensitivity testing determination

Annex 4-Serial dilution and MIC determination

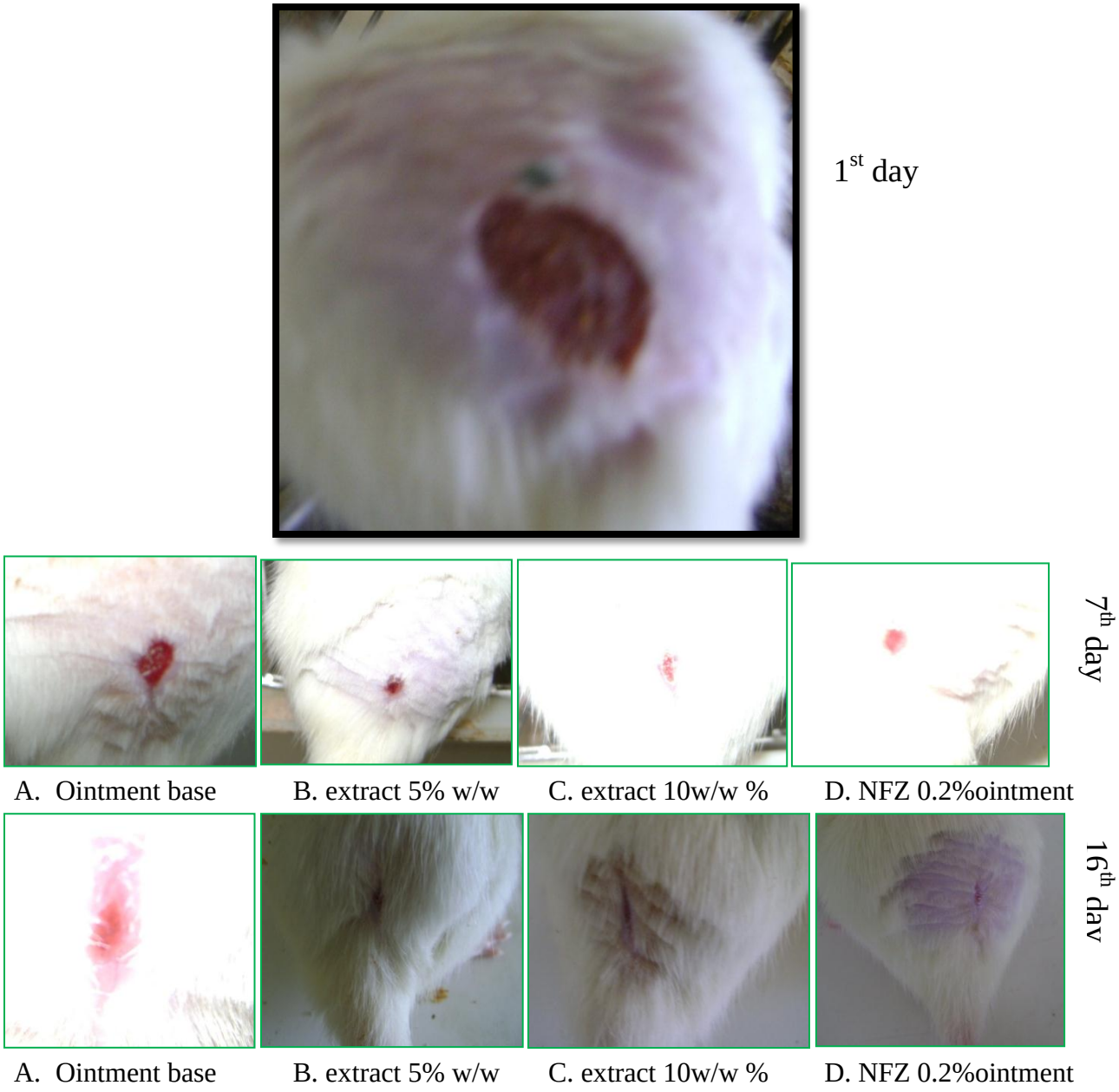

Figure 3.Photographic representation of contraction rate showing percent wound contraction area on different post excision days of ointment base, extract 5% w/w, extract 10w/w % and NFZ 0.2%ointment) treated rats.